The active sites of the β -lactamases of *Streptomyces cacaoi* and *Streptomyces albus* G

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The active-site serine of the extracellular β -lactamases of *Streptomyces cacaoi* and *Streptomyces albus* G has been labelled with β -iodopenicillanate. The determination of the sequence of the labelled peptides obtained after trypsin digestion of the denatured proteins indicate both enzymes to be class A β -lactamases. Surprisingly the two *Streptomyces* enzymes do not appear to be especially homologous, and none of them exhibited a high degree of homology with the *Streptomyces* R61 DD-peptidase. Our data confirm that, as a family of homologous enzymes, class A is rather heterogeneous, with only a small number of conserved residues in all members of the class.

INTRODUCTION

The properties of β -lactamases synthesized and excreted by two strains of *Streptomyces* have been studied in the last few years (Duez *et al.*, 1981; Ogawara *et al.*, 1981; Frère *et al.*, 1982; Lenzini & Frère, 1985). Those enzymes are most active against classical penicillins, but also hydrolyse cephalosporins at a non-negligible rate. Such a substrate profile and their insensitivity to metal-chelating agents distinguish them from classes C and B β -lactamases respectively. Detailed studies of their interactions with β -iodopenicillanate, an inactivator of serine β -lactamases, indicated clear similarities with the general properties of class A enzymes (De Meester *et al.*, 1986), although the inactivation of the *S. cacaoi* β -lactamase was distinctly slower than that of most class A enzymes (Lenzini & Frère, 1985).

Recently, striking similarities were found in the relative positions of most secondary-structure elements of the class A β -lactamases of *Bacillus cereus* and *Bacillus licheniformis* and those of another *Streptomyces* extracellular enzyme: the penicillin-sensitive DD-peptidase of *Streptomyces* R61 (Kelly *et al.*, 1986; Samraoui *et al.*, 1986). Surprisingly, at the primary-structure level, more homologies were found between the DD-peptidase and class C β -lactamases than with the class A enzymes (Joris *et al.*, 1987a; Duez *et al.*, 1987). In consequence, it became extremely important to obtain sequence and structure data on the *Streptomyces* β -lactamases, in order to compare those enzymes with a penicillin-sensitive DD-peptidase produced by bacteria of a closely related species. Parallel studies were thus undertaken to label, isolate and sequence active-site peptides of the two *Streptomyces* β -lactamases and to determine the nucleotide sequences of the corresponding genes. We here present the results obtained by the first approach.

MATERIALS AND METHODS

Enzymes

Tos-Phe-CH₂Cl-treated trypsin was purchased from Millipore Corp. (Freehold, NJ, U.S.A.). The *Streptomyces cacaoi* and the *Streptomyces albus* G β -lactamases were produced by *Streptomyces lividans* after transformation by pIJ702 plasmid derivatives pDML51 and pDML6 respectively (Dehottay *et al.*, 1986; Lenzini *et al.*, 1985). For production of the *S. cacaoi* enzyme, *S. lividans* was grown in E9 medium (Dehottay *et al.*, 1986). After 48 h at 28 °C, the culture filtrate was separated from the mycelium by centrifugation and the enzyme was purified by using a slight modification of the procedure described by Ogawara *et al.* (1981). Similarly, for production of the *S. albus* G enzyme, *S. lividans* was grown in Lennox (1955) medium. After 48 h at 28 °C, the culture filtrate was collected and the enzyme was purified by the procedure described by Duez *et al.* (1981).

Chemicals

Dithiothreitol and Nbs₂ were purchased from Janssen, Beerse, Belgium. The sample of β -iodo[β -methyl-³H]-penicillanic acid (β IP) was that prepared previously (De Meester *et al.*, 1985).

Spectra

U.v. spectra were recorded on a Beckman DU8 or a LKB Ultrospec 4015 spectrophotometer.

Peptide analysis and sequencing

Amino acid compositions after a 24 h hydrolysis of the peptide in azeotropic HCl were obtained by using a D-300 Dionex analyser equipped with a Waters column and Spectra Physics detector. Sequencing was performed on

Abbreviations used: Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid) ('DTNB'); β IP, β -iodopenicillanic acid; f.p.l.c., fast protein liquid chromatography.

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Fig. 1. Purification of the labelled peptide obtained by trypsin digestion of the *S. cacaoi* β -lactamase on the Pro-RPC column using gradient A

A 0.5 ml portion, representing one-third of the total material, was injected. The Figure shows the absorbance profile at 215 nm (—), the radioactivity of the fractions (----) and the gradient (---) reaching 76% of buffer B (see the text) after fraction 50. The radioactivity was determined on portions representing 10% of the volume of each fraction. For other details, see the text.

a 470-A Applied Biosystems gas-phase sequencer as described previously (Joris *et al.*, 1985). *N*-Terminal residues were determined by dansylation (Hartley, 1970).

Peptide purification

This was done with the help of a f.p.l.c. apparatus (Pharmacia, Uppsala, Sweden) equipped with a Pro-RPC column (0.5 cm $\times$ 5 cm). The buffers and gradients were as follows. Buffers A and B contained 10 mM-NH₄HCO₃ respectively in 100% water and in water/acetonitrile (2:2, v/v). Buffers C and D contained 0.1% trifluoroacetic acid respectively in 100% water and in water/acetonitrile (3:7, v/v). Gradient A started with 1 ml of buffer A and went from 0 to 100% of buffer B over 18 ml. Gradient B started with 1 ml of buffer A and went from 0 to 65% of buffer B over 20 ml. Gradient C started with 1 ml of buffer C and went from 0 to 80% of buffer D over 20 ml. In all cases the flow rate was 0.3 ml $\cdot$ min⁻¹ and 0.3 ml fractions were collected.

RESULTS

The active-site peptide of the β -lactamase of *S. cacaoi*

The enzyme (6 mg, i.e. 176 nmol) in 3 ml of 10 mM-Tris/HCl buffer, pH 7.2, containing 10% ethylene glycol was added with 25 μ l of 10 mM-[³H] β IP (inactivator/enzyme ratio 1.4:1). Inactivation was complete after 10 min at 30 $^{\circ}$ C. A u.v. spectrum was recorded, indicating the expected A_{325}/A_{280} ratio of 0.44 (De Meester *et al.*,

Fig. 2. U.v. spectrum of the purified peptide obtained by trypsin digestion of the *S. cacaoi* β -lactamase

The solution contained about 8 nmol of peptide (on the basis of the radioactivity) in 300 μ l of water.

Table 1. Amino acid composition of the active-site peptides

Peptide . . .	<i>S. cacaoi</i>		<i>S. albus</i> G	
	Amount (nmol)	No. of residues*	Amount (nmol)	No. of residues*
Lys	1.8	1	0.69	1
His	—	—	—	—
Arg	—	—	—	—
Asx	—	—	0.57	1
Thr	2.0	1	—	—
Ser	1.7	1	0.68	1
Glx	—	—	0.56	1
Pro	—	—	0.62	1
Gly	2.3	1	0.31	0
Ala	1.5	1	0.64	1
$\frac{1}{2}$ -Cys	—	—	P†	(1) ?
Val	—	—	0.65	1
Met	—	—	0.47	1
Ile	—	—	—	—
Leu	—	—	0.58	1
Tyr	0.9	1	—	—
Phe	3.0	2	1.01	2
Total . . .		8		11–12

* Nearest whole number.

† P, present but not accurately measured.

1986). The sample was dialysed for 12 h against water and the determination of the radioactivity of a portion indicated an inactivator/enzyme ratio of 0.96:1. After freeze-drying, the powder was dissolved in 250 μ l of 100 mM-NH₄HCO₃ containing 100 μ M-CaCl₂ and 8 M-urea. The sample was incubated for 60 min at 37 $^{\circ}$ C, diluted with 400 μ l of the same buffer devoid of urea and, after addition of 600 μ g of trypsin, further incubated at

	62				65				70				73				References
<i>Streptomyces cacaoi</i>	[Ala	Asp	Glu	Arg]	Phe	Ala	Tyr	Gly	Ser*	Thr	Phe	Lys	}	The present study			
<i>Streptomyces albus G</i>	Ala	Asp	Glu	Leu	Phe	Pro	Met	Cys	Ser	Val	Phe	Lys					
<i>Klebsiella pneumoniae</i>					Phe	Ala	Met	Cys	Ser	Thr	Ser	Lys					Joris <i>et al.</i> (1987b)
<i>Klebsiella aerogenes</i>					Phe	Ala	Met	Asn	Ser	Thr	Ser	Lys				Emanuel <i>et al.</i> (1986)	
<i>Bacillus licheniformis</i>	Pro	Asp	Glu	Arg	Phe	Ala	Phe	Ala	Ser	Thr	Ile	Lys	}	Ambler (1980)			
<i>Bacillus cereus</i> I	Pro	Asn	Glu	Arg	Phe	Ala	Phe	Ala	Ser	Thr	Tyr	Lys					
<i>Staphylococcus aureus</i>	Ser	Asp	Lys	Arg	Phe	Ala	Tyr	Ala	Ser	Thr	Ser	Lys					
Plasmid RTEM 2	Pro	Glu	Glu	Arg	Phe	Pro	Met	Met	Ser	Thr	Phe	Lys					
<i>Rhodopseudomonas capsulata</i>	Glu	Asp	Glu	Leu	Phe	Leu	Met	Asn	Ser	Thr	Val	Lys	}	J. I. Campbell & R. P. Ambler, personal communication			
<i>Pseudomonas aeruginosa</i> (plasmid)	Gly	Asp	Glu	Arg	Phe	Pro	Leu	Asn	Ser	Thr	His	Lys					
Class C (consensus)	Gln	Gln	Thr	Leu	Phe	Glu	Leu	Gly	Ser	Val	Ser	Lys					
	Pro	Glu					Ile			Ile			}	Joris <i>et al.</i> (1986)			
	Glu						Val			Leu							
OXA 2	Ser	Lys	Lys	Arg	Tyr	Ser	Pro	Ala	Ser	Thr	Phe	Lys					Dale <i>et al.</i> (1985)
<i>Streptomyces</i> R61 DD-carboxypeptidase	Thr	Thr	Asp	Arg	Phe	Arg	Val	Gly	Ser	Val	Thr	Lys				Duez <i>et al.</i> (1987)	

Fig. 3. Comparison between the sequences of the active-site peptides of the *S. cacaoi* and *S. albus G* β -lactamases and those of corresponding areas of other class A β -lactamases

The highly conserved sequences of class C β -lactamases and those of the OXA-2 enzyme (class A?) and of the *Streptomyces* R61 DD-peptidase are also shown. Residues 62–65 in the *S. cacaoi* enzyme were deduced from the partial nucleotide sequence of the gene (M. V. Lenzini, unpublished work). The numbering is that of Ambler (1980).

37 °C for 3 h. The digest was filtered through a Sephadex G-25 column (100 cm $\times$ 1 cm) in 50 mM-NH₄HCO₃. Two groups of radioactive fractions were detected: the first group (25% of total radioactivity, K_{av} = 0.16) probably corresponded to incompletely digested material and was not further examined. The fractions corresponding to the second group (75%, K_{av} = 0.33) were pooled and freeze-dried. The dry powder was dissolved in 1.5 ml of buffer A and 0.5 ml aliquots were injected into the Pro-RPC column. Fig. 1 shows the elution profile for one of the three runs performed with gradient A. Fractions 35–37 were pooled and freeze-dried. Fig. 2 shows a u.v. spectrum of the peptide after redissolution of the dry powder in water. The N-terminal residue was identified by using 3 nmol of material. Only phenylalanine was found. The amino acid composition after hydrolysis with 6 M-HCl of 1.8 nmol is shown in Table 1. The sequence determined with 8 nmol of material and the gas-phase sequencer is shown in Fig. 3.

Active-site peptide of the β -lactamase of *S. albus G*

In a first experiment, 140 nmol of enzyme (70% purity) in 10 mM-Tris/HCl buffer, pH 7.2, containing 8% glycerol and 8% ethylene glycol was dialysed against 50 mM-sodium phosphate buffer, pH 7.0, containing 1 M-NaCl, 8% glycerol and 8% ethylene glycol. The high

salt concentration was used to decrease the ratio of turnover of β IP to inactivation (see De Meester *et al.*, 1986). To the dialysed solution, 250 μ l of 10 mM-[³H] β IP were added, yielding an inactivator/enzyme ratio of 9.5:1. After 10 min at 30 °C, inactivation was complete and the mixture was exhaustively dialysed against water. The ratio A_{325}/A_{280} was 0.31, in good agreement with previous results when adjusted for the presence of 30% impurity in the initial preparation. Similarly, after the same correction, determination of the radioactivity of a portion indicated a bound inactivator/enzyme ratio of 0.98:1. The sample was freeze-dried and the powder dissolved in 300 μ l of 100 mM-NH₄HCO₃, containing 0.1 mM-CaCl₂ and 8 M-urea. The solution was incubated for 2 h at 37 °C and 300 μ l of the same buffer, devoid of urea, were added, together with 500 μ l of trypsin. The mixture was incubated for 5 h at 37 °C. The digest was filtered through the Sephadex G-25 column, but most of the radioactivity was excluded. A second filtration was then performed through a Sephadex G-50 column (150 cm $\times$ 1 cm). The radioactive fractions, centred at a K_{av} value of 0.2, were pooled and freeze-dried. The powder was dissolved in 1.5 ml of 10 mM-NH₄HCO₃; the sample was injected in the Pro-RPC column (three runs) and was eluted with gradient B. Two major radioactive peaks were observed (Fig. 4). The corresponding fractions

Fig. 4. Chromatographic profile of the first trypsin digest of the *S. albus* G β -lactamase on the Pro-RPC column using gradient B

A 0.5 ml aliquot, representing one-third of the total material, was injected. The gradient (---) reached 56% of B after fraction 63. For conditions and symbols, see the legend to Fig. 1. The contents of fractions 30–32 (peptide AG3) were also analysed. Only one *N*-terminal residue (leucine) was found.

were pooled and the *N*-terminal residue determined for each group by using 1 nmol of peptide (on the basis of the radioactivity). For peptide AG2, alanine was found as the unique *N*-terminal residue. For peptide AG1, several residues were found (including alanine), and the peptide was not further analysed. The sequence determined on 2 nmol of peptide AG2 is given in Fig. 3. However, the amino acid composition (not shown) did not exactly correspond to this sequence and, on sequencing, a second peptide, representing about 20% of the major one, was consistently found. This peptide appeared to be larger than the peptide presented in Fig. 3, since a sharp decline in the total yield was observed after the 12th residue of the major peptide and several residues were still detected with a similarly low yield. Difficulties had been encountered before with a cysteine-containing peptide from *Klebsiella pneumoniae* (Joris *et al.*, 1978b), and we decided to perform a second digestion after blocking the free thiol(s) rather than trying to further purify peptide AG2.

A second experiment was performed where 63 nmol of enzyme in 1 ml of 50 mM-sodium phosphate, pH 7.0, containing 1 M-NaCl, 8% glycerol and 8% ethylene glycol were inactivated by 500 nmol of [3 H] β IP. After dialysis and freeze-drying, the powder was dissolved in 2 ml of 8 M-urea (in water), containing 0.7 mM-dithiothreitol, and the solution was incubated at 37 °C for 4 h. A 10 mM solution of Nbs₂ was then added to yield a final concentration of 1.5 mM, and the mixture was incubated at 37 °C for 30 min. The solution was exhaustively dialysed against water and freeze-dried. The powder was dissolved in 500 μ l of 100 mM-NH₄HCO₃,

Fig. 5. Purification of the peptide labelled with both Nbs₂ and β IP on the Pro-RPC column using gradient C

Only one run was performed. The gradient (---) reached 86% of buffer D (see the text) after 60 fractions. For conditions and symbols, see the legend to Fig. 1.

Fig. 6. U.v. spectra of the peptide labelled with both Nbs₂ and β IP before (a) and after (b) addition of 0.33 mM (final concn.)-mercaptoethanol

containing 100 μ M-CaCl₂ and 8 M-urea, and the solution incubated at 37 °C for 2 h. Trypsin (150 μ g in 0.5 ml of water) was then added and the sample was further incubated for 5 h at 37 °C, and filtered through the Sephadex G-50 column. The radioactive fractions were pooled, freeze-dried, and the powder was dissolved in 600 μ l of water. The sample was injected on the Pro-RPC column and eluted with gradient C (Fig. 5). Fig. 6(a) shows the u.v. spectrum of fraction 45 after freeze-drying and redissolution in 300 μ l of water. The sample was then added with 0.33 mM-mercaptoethanol (final concn.), and a new spectrum was recorded after 10 min at 20 °C. Fig. 6(b) clearly shows a decrease of A_{318} and the appearance of a new chromophore centred at 416 nm and corresponding to free Nbs²⁻ (5-thio-2-nitrobenzoate).

The sample (fraction 45) was dry-evaporated and hydrolysed with 6 M-HCl. The results of the amino acid analysis given in Table 1 confirmed the composition of the peptide whose sequence is given in Fig. 3. The same sequence was obtained by determination of the deoxy-nucleotide sequence of the gene coding for the enzyme (P. Dehottay, unpublished work).

DISCUSSION

The most striking characteristic of both *Streptomyces* peptides is the presence of the sequence

around the active-site serine residue (Ser*; Fig. 3). This sequence has been observed in all β -lactamases studied so far, with the sole exception of the OXA-2 enzyme, where

phenylalanine is replaced by tyrosine (but that could be considered as a minor change), and corresponds to residues 66–73 in Ambler's (1980) numbering. The general comparison of all known β -lactamases sequences around the active-site serine residue depicted in Fig. 3 establishes that the *Streptomyces cacaoi* enzyme is a member of class A and strongly suggests that the *S. albus* G enzyme may also be so, in agreement with the predictions made on the basis of the interactions between those enzymes and β IP. The *S. cacaoi* β -lactamase appears as a rather conventional class A enzyme. The Asp-Glu-Arg (63–65) sequence, residues Ala-67 and Thr-71, and aromatic residues in positions 68 and 72 are observed in several other class A enzymes. Only one residue (Gly-69) is typical of class C enzymes and is also found in a homologous position in the R61 DD-peptidase.

In contrast, the *S. albus* G β -lactamase has two typical class C residues: the most striking is Leu-65, which occupies a position where arginine is found in all class A enzymes (with the exception of the *Rhodopseudomonas capsulata* enzyme), but also in OXA 2 and the DD-peptidase. Val-71 is also a class C characteristic, which in this case is also shared by the DD-peptidase. However, Asp-63, Glu-64, Met-68 and an aromatic residue in position 72 indicate that the *S. albus* G enzyme belongs to class A. While this work was in progress, nucleotide sequences of the genes coding for both *Streptomyces* enzymes were obtained, which confirmed the homology with class A enzymes (M. V. Lenzini & P. Dehottay, unpublished work).

Our data underline another characteristic of class A: its heterogeneity. Indeed, if one excepts Phe-66, Ser-70 and Lys-73 (which are also present in all other enzymes), no 'consensus' sequence of class A can be written. At all positions there is always at least one enzyme which exhibits an individual behaviour, for instance Leu-65 in *S. albus* G and *R. capsulata* (in all other other enzymes, arginine), Leu-67 in *R. capsulata* (alanine or proline in the others), Leu-68 in *Pseudomonas* (tyrosine, phenylalanine or methionine in the others) and Val-71 in *S. albus* G (threonine in the others). Some positions are highly variable in class A enzymes, where the substitutions are certainly not conservative: positions 62 (Ala, Pro, Ser, Gly or Glu), 69 (Gly, Cys, Asn, Ala, Met) and 72 (Phe, Ser, Ile, Tyr, Val, His). It should be noted that, in class C, many substitutions are highly conservative (positions 68 and 71 for instance).

It is also apparent, and rather surprising, that no marked homology is observed between the two *Streptomyces* β -lactamases, in contrast with the two *Bacillus* enzymes. Indeed, a stronger homology is found between the *S. cacaoi* and the *B. licheniformis* enzymes than between the two *Streptomyces* enzymes. An interesting characteristic of the *S. albus* G enzyme, shared only by the *Klebsiella pneumoniae* β -lactamase, is the occurrence of a cysteine residue next to the active-site serine residue. Various data (Joris *et al.*, 1987b; P. Dehottay, unpublished work) indicate that the residue might be deeply buried in these enzymes.

When the comparison is extended to the OXA 2 β -lactamase and to the R61 DD-peptidase, it seems that the first enzyme is much closer to class A, which justifies its classification as a 'honorary' class A member (the term was coined by S. G. Waley). In contrast, and as discussed elsewhere, the R61 DD-peptidase is distinctly closer to class C enzymes (Duez *et al.*, 1987), but it is also

noteworthy that more similarities are found between the peptidase and class A than between the two classes of β -lactamases. When the sequences depicted in Fig. 3 (i.e. 17 proteins, since five class C enzymes are known) are compared pairwise, identities vary from 2 to 12 residues out of 12 aligned (the best score has been obtained with two class C enzymes). If the OXA-2 β -lactamase is not included, Phe-66, Ser-70 and Lys-73 are always conserved, resulting in a minimum of 25% identity. However, as noted before, the homology between the various groups of enzymes becomes much less distinct when the complete sequences are compared. One can only hope that X-ray-diffraction studies will, in the not-too-distant future, show the three-dimensional arrangement of those 12 residues and whether, in addition to the active serine residue, the conserved phenylalanine (tyrosine in OXA 2) and lysine residues are found in equivalent positions so that they can fulfil similar functions.

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